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STUDY OF TOXIC AND ANTIGENIC STRUCTURES OF BOTULINUM NEUROTOXINS (U)

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Procedure to purify types A, B and E neurotoxin (NT) using FPLC was developed. The NT produced by strain B-657 was identified and characterized based on partial amino acid sequence. The heavy chains of types A, B and E NTs were enzymatically cleaved at about the mid-point. The two halves of the heavy chain of type A were isolated and partially sequenced. Specific chemical modification reactions were used to examine the role of carboxyl groups, lysine and tyrosine residues. Type B and E NTs completely detoxified following tyrosine modification were used as immunogen to raise, in rabbits, NT neutralizing antibody. Conformation of the NTs and the isolated heavy and light chains were examined by circular dichroism, fluorescence spectra and UV-difference spectra. Interaction of the subunit chains of the three NTs with lipid bilayer surface was examined. Keywords: Clostridium Botulinum, Amino acid Sequence. (AW)

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18: generation toxoid, RA 1

## Summary

An automated modern chromatographic system, Fast Liquid Chromatography, was employed to purify types A, B and E botulinum neurotoxin (NT). Purification of types A and B NT by an automated system was accomplished for the first time. The type B NT isolated from Clostridium botulinum (strain 657) was significantly purified. The NT behaved as a mixture of single (unnicked) and dichain (nicked) protein both of $M_r$ ~150K. The two chains, separable in SDS-PAGE, were similar to the H and L chains of other serotypes of botulinum NT. The N-terminal sequences of the two chains were partially determined. These two sequences matched exactly with those of the H and L chains of type B NT (strain Okra). Two enzymatic (trypsin) procedures were developed to cut the H chain of type A NT at about the midpoint, and to purify the two halves of the H chain. Partial amino acid sequence of the C-terminal half was determined. Roles of carboxyl groups, lysine and tyrosine residues in types A, B, E NTs (with respect to their structure-function relationship) were studied based on chemical modification, quantitative assay of amino acid modification, toxicity and change in serological activity. Pure type B NT completely detoxified following modification of tyrosine residues was found to be a good immunogen (second generation toxoid) in rabbits. The antiserum had high neutralizing titer for the homologous NT. Examination of the ionization of phenolic groups of tyrosine residues of dichain type A, E and single chain type E NT has detected conformational changes resulting from nicking the single chain to the dichain protein; thus the dichain type E NT appears more like dichain type A NT. Conformations of dichain type A (nicked by endogenous protease), single chain type E and dichain type E (nicked by trypsin) were compared at different pH values using circular dichroism and fluorescence spectroscopy. The secondary and tertiary structures of type A NT and the state of tyrosine and tryptophan residues were determined. In the dichain type A NT the H and L chains appear to retain two quasi-independent structural domains although connected by -S-S- bond(s). The secondary structures of the subunit chains do not significantly change when they are separated after breaking the -S-S- and noncovalent bonds linking them. Both the H and L chains of type A, B and E NTs were found to attach to the lipid bilayer surface.

## FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS Publication No. (NIH) 86-23, Revised 1985).

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## Body of the report

### Problems and challenges:

The ubiquitous spore forming bacteria Clostridium botulinum produce the protein, botulinum neurotoxin (NT). It is the sole cause of the neuroparalytic disease botulism. The NT blocks release of the neurotramsmitter acetylcholine (ACh) from the presynapses at neuromuscular junctions. The consequent flaccid muscle paralysis can lead to asphyxiation and death. Botulism is generally caused by ingestion of the NT. In improperly preserved food, spores germinate with subsequent multiplication of the bacteria and NT production (1). Cases of botulism have resulted from wounds (1). A puzzling aspect of the disease is toxico-infection in humans of ages 3-35 weeks. It is called infant botulism and results from ingestion of spores and not the preformed NT (2).

The bacteria produce the NT in antigenically distinct forms called types A, B, $C_1$, D, E, F and G. In general, a pure culture produces one immunological type of NT, e.g. type A NT is produced by type A strain. Until about 1960 only types A-E were known. Since then recognition of types F and then G (3) indicates that other unrecognized types may be lurking in nature, to which we are openly vulnerable. Within the past 10 years one unusual type B NT (from strain 657, isolated from human infant botulism) was identified. It is ~5000 times more toxic to infant mice than adult mice, and requires ~1000 times more standard type B antiserum for neutralization than the well-known type B NT (4).

These facts show our poor understanding of the immunogenic/antigenic nature of the NT. And yet, definitive diagnosis and the only effective therapy for botulism depends, at present, on a chemically modified form(s) of the NT i.e. toxoid (immunogen) and the antiserum raised with the immunogen.

Little is known about the structure and less about the antigenic/immunogenic characteristics of the NT. The NT is a single chain (unnicked), a dichain (nicked) molecule or a mixture of single and dichain molecules. The two chains are held together by at least one -S-S- bond and by weak non-covalent bonds. The mol. wt. of heavy (or H) chain, ~100,000, is twice the weight of the light (or L) chain. All NT types are of mol. wt. ~150,000 (5). The single chain molecule is nicked endogenously into the dichain form in cultures of some (e.g. type A) but not in all types (e.g. type E). Mild trypsinization of any single chain NT results in its nicking and also in the 10-500 fold increase in its specific toxicity (5). The H and L chains of types A (6) and B (7) NT were separated and the individual chains appeared non-toxic. When the separated H and L chains of type A (6) or B (7) were brought together, the -S-S- bond formed between the two chains, and the reconstituted NT (mol. wt. 150,000) regained toxicity. This means that in vivo toxicity (mouse lethality) depends on the cooperative action of the complementary chains. The amino acid compositions of dichain NT types A, B and E, their H and L chains and the partial amino acid sequences of these three NTs are now known (8).

The toxoid(s) currently used for immunization of humans and animals is a very crude preparation of the NT (~90% impure) detoxified with formaldehyde (9,10). The toxoid, a very heterogenous and poorly defined product, can be considered as the first generation toxoid. The active component, the NT, undergoes extensive modification (modification of 10-25% of Thr, Met, Tyr and Lys residues and polymerization, DasGupta, unpublished) in the process of losing toxicity and therefore has potential drawbacks as an immunogen. This point can be further extended by asking the question: if only a few amino acid residues of the NT, critical for toxicity (e.g., for binding, translocation or paralytic activity--the three steps in the mode of action of the NT) are modified to detoxify the NT completely, would the resultant detoxified protein act as an immunogen, better than the toxoid prepared with formaldehyde (which causes more drastic change in the protein)?

The available toxoids contain formaldehyde to prevent the toxoid from reverting back to a partially toxic form in the absence of the reagent (11). Modification of specific amino acids could produce detoxified protein that would not revert back to a toxic form when the detoxifying reagent is removed from the reaction mixture. Thus, unlike the case of toxoid plus formaldehyde, one can have the detoxified protein free of the detoxifying agent. Stable covalent modification of critical amino acid residues would produce this toxoid. Design and development of these second generation toxoids made of ~99% pure NT detoxified by selective modification of a few amino acid residues was undertaken.

Antigenicity/immunogenicity of the NT was studied based on the chemically modified NT and polyclonal antisera. When modification of certain amino acid residue, e.g. Lys did not reduce serological reactivity of the NT the Lys residues were thought not critical for antigenicity. On the other hand, when modification of another kind of residue, e.g. Tyr lead to reduced serological reactivity Tyr residues were thought to be critical for antigenic structures. This approach has certain limitations because the observed effect is the sum of serological reactions between all the epitopes on the NT and the corresponding many antibodies in the polyclonal serum.

Two kinds of antigenic determinants were considered: 1) "A segmental (continuous) site exists wholly within a continuous segment of amino acid sequence. 2) An assembled topographic site consists of amino acid residues far apart in the primary sequence but brought together in the surface topography of the native protein by the way it folds in three dimensions" (12).

The planned chemical modifications of amino acid residues of a NT might also alter the conformation of the NT. The associated altered biological activity might not result directly from the modification but might result from change in the conformation. These questions are difficult to answer at this stage of the work.

Two requirements for understanding the antigenic structures of the NTs are their primary structures and conformations. The amino acid sequence determination (Edman degradation) followed separation of the subunits of the NTs and also cleavage of the large NT molecules at precise sites and isolation of the small fragments. Conformations were deduced from spectrophotometric and fluorometric techniques. Nothing was known about the secondary and tertiary structure of the NT.

The NT elaborated by the strain B-657 was purified for partial characterization and compared with the well known type B NT produced by strain Okra. The binding of the two subunits of the NT to lipid surface was examined to learn their role in toxicity and therefore indirectly their immunogenicity.

The entire study consists of separate and independent approaches. Hence the rest of this report is presented as nine separate sections each with its experimental methods, results, discussion and conclusions.

## 1. FAST PROTEIN LIQUID CHROMATOGRAPHY OF NEUROTOXIN TYPES A, B AND E:

In order to derive the benefits of an automated modern chromatographic system the Fast Protein Liquid Chromatography was employed to: i) examine whether isolation of a NT from its complex can be made more efficient (operation time, yield and purity of NT); and ii) establish the chromatographic parameters of NT. Three antigenically different NTs ($M_r$ ~150,000), classically distinguished only by specific antisera, were for the first time chromatographically resolved. Mixed NTs eluted from the Mono Q column in order of types E, A and B, and from the Mono S column as B, E and A. Types A and B NTs were successfully chromatographed on the cation exchanger Mono S column above their isoelectric points. Purification of types A and B NTs by automated liquid chromatography was also accomplished for the first time. Types A, B and E NTs were purified by application on anion-exchanger Mono Q, followed by use of cation-exchanger Mono S column. See publication #1.

## 2. CHARACTERIZATION OF THE NEUROTOXIN FROM TYPE B STRAIN 657:

The strain 657, isolated from a case of human infant botulinum, produced type B toxin that appeared unusual in its serological properties; at least two species of toxic proteins were thought to be involved of which only one corresponds to the type B specificity (Hatheway et al. J. Clin. Microbiol. 14, 607 [1981]). It was also reported that the 657 toxin is a mixture of type B (~95%) and type A toxins and the type B toxin from strain 657 represents the prototype of a new serotype named subtype Ba (Gimenez Zbl. Bakt. Hyg. A. 257, 68 [1984]). This conclusion was based on cross-neutralization tests done with anti-serum prepared against neurotoxin serotypes A, B and B-657.

We purified the 657 toxin following a method of purification of type A rather than that of type B (strain Okra) neurotoxin (NT). Following its purification, the 657 toxin was identified serologically by testing against antitype A and antitype B (strain Okra) NT sera. Also the two chains of the 657 toxin were separated and analyzed for amino acid sequence.

The toxin behaved as a mixture of single (unnicked) and dichain (nicked) protein, both of $M_r$~150K. When the protein was reduced with mercaptoethanol the two chains migrated in SDS-PAGE as separate polypeptides of $M_r$ ~100K and 50K that appeared similar to the heavy and light chains of other serotypes of botulinum NT. The N-terminal amino acid sequences of the two chains were determined. They were as follows; light chain: Pro.Val.Thr.Ile.Asn.Asn.Phe.Asn.Tyr.Asn.Asp.Pro.Ile.Asp.Asn.Asn.Asn.Ile.Ile.

Met.Met.Glu.Pro.Pro.Phe.Ala.Arg.Gly.Met.Gly.Arg.Tyr.Tyr.Lys.Ala.Phe.Lys.Ile.Thr.Asp.Arg.Ile.Trp.Ile.-; and heavy chain: Ala.Pro.Gly.Ile.X.Ile.Asp.Val.Asp.Asn.Glu.Asp.Leu.Phe.Phe.Ile.Ala.Asp.Lys.Asn.Ser.Phe.Arg.Asp.Asp.Leu.- These two sequences matched exactly with those of the light and heavy chains of type B NT (strain Okra) of which only 16 and 18 residues were known (Sathyamoorthy and DasGupta J. Biol. Chem. 260, 10461, 1985). The above sequences were different from those of type A NT. Immunoprecipitation reactions of type B NT isolated from strains 657 and Okra were indistinguishable against polyclonal antitype B NT serum. These two preparations did not produce precipitin reactions with polyclonal antitype A NT serum. The tetanus NT (from Clostridium tetani) resembles botulinum NT in relative molecular mass, dichain structure and probably in the mode of action; but the two NTs differ in their sites of action and their serologic specificity. Comparison of amino acid sequences of type B botulinum NT, reported here, and tetanus NT (Eisel et al. EMBO J. 5, 2495, 1986) shows that their two heavy chains are less homologous (13 out of 26 residues matched in position) than the two light chains (31 out of 44 residues matched). Three stretches residues #3-7, 18-24 and 32-44 on the light chains of the two NTs are identical. See publication #2.

3. ENZYMATIC CLEAVAGE OF THE HEAVY CHAIN AND PARTIAL CHARACTERIZATION OF THE TWO FRAGMENTS:

The two halves, i.e. the N- and C-terminal halves, of the heavy chain of the NT is thought to have different functions in the mechanism of action of the NT (by analogy with diphtheria and tetanus toxin). Well characterized and highly purified preparations of the two halves of the heavy chain are needed for such studies. Two different approaches were taken to cut the heavy chain with trypsin and isolate the fragments. In one method, the intact type A NT (150 KDa) was digested with trypsin to cut the heavy chain. The cleavage products were: i) 94 KDa fragment made of the L chain linked to the N-terminal half of the heavy chain (49 KDa) by a disulfide bond(s), and ii) the C-terminal 44 KDa fragment. The second method developed was as follows: The heavy chain of type A NT was first separated from the light chain, purified and then digested with trypsin. One product of cleavage, the 44 KDa fragment (C-fragment) was chromatographically purified and then analyzed for amino acid composition and sequence. The sequence, given below, matches with the sequence of the C-fragment (only 12 residues were sequenced) obtained by method one (i.e., digestion of intact NT) except at positions 1 and 3.

```
1       5         10        15        20
S-I-R-N-L-G-I-L-N-L-R-Y-E-V-N-D-L-I-D-L-R-Y-Y-H-
```

This work also demonstrated that in type A i) the cysteine residues located on the N-terminal half of the heavy chain forms the -S-S- link(s) with the light chain. ii) The C-terminal half (44 KDa fragment) is not linked via -S-S- to the L-chain or to the N-terminal half (49 KDa fragment) of the heavy chain.

Digestion of intact type E NT with trypsin also cleaved the heavy chain at about the mid-point. One cleavage product was the light chain linked to the N-terminal half of the heavy chain via disulfide bond(s), hence its total size

~100 KDa. This fragment of the heavy chain was separated from the light chain, chromatographically purified and partially sequenced. Two independent sequence runs gave a sequence (see below) which is identical to the known N-terminal sequence of the intact heavy chain. This confirms that the isolated and purified fragment is the N-terminal half of the heavy chain.

```
1       5         10
K-S-I-C-I-E-I-N-N-G-E-L-F-
```

Tryptic fragmentation at about the mid-point of the heavy chain of type B NT has been accomplished at the analytical level. See publication #3.

4. STRUCTURE-FUNCTION RELATIONSHIP:

The role of amino acid residues as structural components of biologically active sites of the NT were examined by specific chemical modification of carboxyl groups, lysine and tyrosine residues.

Carboxyl Groups: A water soluble carbodiimide-nucleophile reaction, that is highly specific for modifying carboxyl groups of proteins was used to study type A and E NTs. In both types A and E increasing levels of the reagents, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and norleucine methyl ester or glycine methyl ester, caused increased loss of toxicity. Amino acid analysis did not reveal modification of any amino acid residue other than carboxyl groups (possible reaction of -SH was not studied). Loss of one carboxyl group did not severely affect toxicity, but modification of three carboxyl groups caused >95% detoxification in both types. Complete detoxification could not be achieved with any amount of the reagents. Modification of a few carboxyl groups did not affect serological activity. See publication #4.

Lysine: Type A and B NTs were modified by reductive methylation using $NaBH_4$ and formaldehyde at different molar ratios of the protein and the reagent and then they were analyzed for change in toxicity, serological reactivity and the number of lysine residues modified. Amino acid analysis of the modified proteins (HCl hydrolysed) confirmed selective modification of lysine. The derivative N,N-dimethyl lysine was more abundant than monomethyl lysine; trimethyl lysine was not detected. Distribution of modified lysine residues among the heavy and light chains of the dichain type A NT was approximately proportional to the lysine contents of the two subunits of the NT. Toxicity (mouse lethality) and serological reactivity (polyclonal antibody) of type A NT were not (or insignificantly) damaged following methylation of up to 72 lysine residues. Modification of 3 additional residues caused precipitious loss in toxicity. The type B NT appeared more susceptible to the effect of lysine modification than type A NT. When 31, 55, 77 or 88 lysine residues of type B NT (out of a total of 118 residues) were modified, mouse death time (31 min for control) was found to be 47, 79, 143 or 186 min, respectively. Difference between type A and B NTs with respect to the effect of modification on toxicity suggests that role of lysine residues in structure-function relationship in these two antigenically distinct proteins may not be identical. See publication #5.

Tyrosine. Type A, B and E NTs were modified at pH 7.9 with tetranitromethane, a reagent highly specific for tyrosine residues. Modification of only tyrosine residues to nitrotyrosine was evident from amino acid analysis of the acid hydrolysates of the modified proteins. The type B and E NTs could be completely detoxified without causing significant damage to their serological reactivities. Under similar modification reaction conditions, the type A NT was incompletely detoxified with some alteration in this serological reactivity. Following relationship between molar excess of reagent over NT, number of nitrotyrosine residues formed and loss in toxicity emerged:

| TNM molar excess | Type A NT | | Type B NT | | Type E NT | |
|---|---|---|---|---|---|---|
| | Nitro-Tyr.* | % loss toxicity | Nitro-Tyr. | % loss toxicity | Nitro-Tyr. | % loss toxicity |
| 500 | 19.7 | <100 | ND | 100 | 24.5 | 100 |
| 125 | -- | 99 | -- | -- | -- | -- |
| 100 | ND | 95 | <1.0 | >98 | 7.6 | >99 |
| 80 | -- | -- | 1.2 (?) | 91 | -- | -- |
| 50 | ND | 85 | -- | -- | -- | >90 |
| 40 | -- | -- | trace | 70 | -- | -- |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* - number of nitrotyrosine residues detected
ND - not detectable, Number of Tyr residues in types A, B, and E NTs are 71, 81, 70, respectively.

See publication #6.

5. SECOND GENERATION TOXOID:

The completely detoxified type B and E NTs, used as toxoid, elicited antibodies in rabbits. The antiserum precipitated and neutralized the homologous NT. Two separate batches of each of these toxoids were prepared for immunizing rabbits in separate groups. The two toxoids, type B and E, were prepared with >99% pure NTs as tested by SDS-PAGE whereas the traditional toxoids produced with formaldehyde are very crude preparations of the NT (~90% impure). Chemical modification induced by tetranitromethane is more specific than the products that form during ~7 days of reaction between a protein and formaldehyde. The toxoids produced with tetranitromethane may be considered as a second-generation toxoid, compared with the first-generation toxoid (crude preparation of NT detoxified with formaldehyde). See publication #6.

6. pH INDUCED DIFFERENCE SPECTRA OF BOTULINUM NEUROTOXIN TYPES A, B AND E:

To understand the role of amino acid residues in the structure and function of the neurotoxin (NT) we examined the state of tyrosine residues based on ionization of phenolic groups and UV difference spectra.

The alkaline pH induced difference spectra (270-310 nm) of three antigenically distinct forms of the botulinum NT types A, B and E were examined. When isolated from the cultures of Clostridium botulinum, type A NT is a fully toxic dichain (nicked) protein, type E is a mildly toxic single chain (unnicked) protein, and type B NT is a mixture of single and dichain proteins and near fully toxic. Trypsin nicks the single chain protein to the dichain and increases its toxicity (up to about 100 fold in type E). A strong difference spectrum peak at ~296 nm was found when types A, B or E NTs were in the alkaline pH region. This peak was not observed at pH 4.0. For types A and B NTs plots of difference absorptivity vs. pH were simple sigmoidal curves. The pK of phenolic moieties of tyrosine residues in both proteins were 10.9. Nearly all tyrosine residues in both proteins were ionized. The single chain type E, unlike type A and B NT, yielded a two step titration curve and pK values 11.3 and less than 7.5; about 60% of the total tyrosine residues present were ionized. The two step titration curve was not observed when the single chain protein was nicked with trypsin to the dichain type E NT. The titration curve of dichain type E NT, although complex, was more like those of type A and B NT. See publication #7.

7. TERTIARY STRUCTURE OF NT:

The conformations of dichain type A (nicked by endogenous protease), single chain type E, and dichain type E NT (nicked by trypsin) were compared at different pHs using circular dichroism (CD) and fluroescence spectroscopy. The high degree of ordered secondary structure ($\alpha$ helix 28%, $\beta$ sheet 42%, total 70%) found in type A NT at pH 6.0 was similar to that found at pH 9.0 ($\alpha$ 22%, $\beta$ 47%, total 69%). The secondary structure of the single chain type E NT at pH 6.0 ($\alpha$ 18%, $\beta$ 37%, total 55%) differed somewhat from these values at pH 9.0 ($\alpha$ 22%, $\beta$ 43%, total 65%). The dichain type E NT at pH 6.0 assumed a secondary structure ($\alpha$ 20%, $\beta$ 47%, total 67%) that seemed more similar to that of dichain type A than the single chain type E NT. Examination with the fluorogenic probe toluidine napthalene sulfonate revealed that the hydrophobicity of the type A and E NTs were higher at pH 9.0 than at pH 6.0. Also, the hydrophobicity of the dichain type E NT was higher than its precursor the single chain protein and appeared similar to that of the dichain type A NT. The CD and fluorescence studies indicate that conversion of the single chain type E NT to the dichain form (i.e. nicking by trypsin) induced changes in conformation. See publication #8.

8. CONFORMATION OF LIGHT AND HEAVY CHAINS OF TYPE A NEUROTOXIN:

The secondary and tertiary structural features of botulinum NT serotype A, a dichain protein ($M_r$ 145,000), and its two subunits, the heavy (H) and light (L) chains ($M_r$ 97,000 and 53,000, respectively) were examined using circular dichroism and fluorescence spectroscopy. Nearly 70% of the amino

acid residues in each of the three polypeptide preparations were found in ordered structure (sum of α helix, β-sheet and β turns). Also, the α-helix, β-sheet, β-turns and random coil contents of the dichain NT were nearly equal to the weighted mean of each of these secondary structure parameters of the L and H chains; e.g., sum of α helix of L chain (22%) and H chain (18.7%), as weighted mean, 19.8% was similar to that of NT (20%) (see table below). These agreements suggested that the secondary structures of the subunits of the dichain NT do not significantly change when they are separated as isolated L and H chains. Fluorescence emission maximum of L chain, 4 nm less (blue shift) than that of H chain, suggested relatively more hydrophobic environment of fluorescent tryptophan residue(s) of L chain. Tryptophan fluorescence quantum yields for L chain, H chain and the NT, 0.072, 0.174 and 0.197, respectively, suggested that: i) an alteration in the micro-environment of the tryptophan residues was possibly caused by interactions of L and H chain subunits of the NT; and ii) quantum yields for L and H chains were altered when they are together as subunits of the NT.

Secondary structural parameters of L chain, H chain and the A NT calculated from their respective far UV circular dichroic spectra between 240 and 200 nm.

| Proteins | α-helix, % | β-sheet, % | β-turn, % | Random coil % |
|---|---|---|---|---|
| A NT | 20.00* | 37.50 | 15.25 | 27.25 |
| L chain | 22.00 | 27.50 | 18.75 | 31.75 |
| H chain | 18.75[a] | 40.00 | 13.00 | 28.25 |
| Weighted mean** | 19.83 | 35.83 | 14.92 | 29.42 |

**Calculated as (1 x L chain + 2 x H chain)/3 because H chain is twice the size of the L chain.

See publication #9.

9. INTERACTION OF THE NEUROTOXIN WITH LIPID BILAYER SURFACE:

The interaction of botulinum NT types A, B and E with the surface of liposomes made of different lipid compositions was studied by photolabelling with a radioiodinated photoactive phosphatidylethanolamine analogue [$^{125}$I-dipalmitoyl (3,4-azidosalicylamido)phosphatidylethanolamine]. When the vesicles were made of negatively charged lipids (asolectin), each of these neurotoxic proteins was radioiodinated, thus providing evidence for their attachment to the membrane surface. The presence of gangliosides on liposome membranes enhanced fixation of the neurotoxic proteins to the lipid vesicle surface. Both the heavy and light chains of the NTs were involved in the

attachment to the lipid bilayer surface. Each of the NTs tested here attached poorly to liposomes made of zwitterionic lipids (egg phosphatidylcholine), even when polysialogangliosides were present. The data suggest that the binding of NTs to their target neuronal cells involves negatively charged lipids and polysialogangliosides on the cell membrane. See publication #10.

Patent report: No patentable information was generated.

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Literature Cited


1. Smith, L. DS. Botulism, The Organism, Its Toxins, The Disease. Charles C. Thomas Publishers, Springfield, IL (1977)

2. Arnon, S. S. Infant botulism, Ann. Rev. Med. 31, 541 (1980)

3. Sakaguchi, G. Clostridium botulinum toxins. Pharmacol. Ther. 19, 165 (1983)

4. Hatheway, C. L., McCroskey, L. M., Lombard, G. L. and Dowell, V. R. Atypical toxin variant of Clostridium botulinum type B associated with infant botulism. J. Clin. Microbiol. 14, 607 (1981)

5. DasGupta, B. R. and Sugiyama, H. Biochemistry and pharmacology of botulinum and tetanus neurotoxins, in Perspectives in Toxinology, (Bernheimer, A. W. ed.) John Wiley & Sons, NY, Chap. 5 (1977)

6. Kozaki, S., Togashi, S. and Sakaguchi, G. Separation of Clostridium botulinum type A derivative toxin into two fragments. Jap. J. Med. Sci. 34, 61 (1981)

7. Kozaki, S., Miyazaki, S., and Sakaguchi, G. Development of antitoxin with each of two complementary fragments of C. botulinum type B derivative toxin. Infect. Immun. 18, 761 (1977)

8. Sathyamoorthy, V. and DasGupta, B. R. Separation, purification, partial characterization and comparison of the heavy and light chains of botulinum neurotoxin types A, B and E. J. Biol. Chem. 260, 10461 (1985)

9. Cardella, M. A. Botulinum toxoids. In: Botulism, proceedings of a symposium (Lewis, K. H. and Cassel, K. Jr. eds). p. 113. U.S. Public Health Service Publication No., 999-FP-1. Public Health Service, Cincinnati, OH (1964)

10. Bowmer, E. J. Preparation and assay of the international standards for Clostridium botulinum types A, B, C, D and E antitoxins. Bull. Wld. Hlth. Org. 29, 701 (1963).

11. Akama, K., Ito, A., Yamamoto, A. and Sadahiro, S. Reversion of toxicity of tetanus toxoid. Jap. J. Med. Sci. Biol. 24, 181 (1971)

12. Berzofsky, J. A. Intrinsic and extrinsic factors in protein antigenic structure. Science 229, 932 (1985)

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1. Woody, M. A. and DasGupta, B. R. Fast Protein liquid chromatography of botulinum neurotoxin types A, B and E. J. Chromatography (Biomedical applications), 430, 279-289 (1988).

2. DasGupta, B. R. and Datta, A. Botulinum neurotoxin type B (strain 657); partial sequence and similarity with tetanus toxin. Biochimie, 70, 811-817 (1988).

3. Sathyamoorthy, V., DasGupta, B. R., Foley, J. and Niece, R. L. Botulinum neurotoxin type A: cleavage of the heavy chain into two halves and their partial sequences. Arch. Biochem. Biophys. 266, (1988).

4. Woody, M., Herian, A. and DasGupta, B. R. Carbodiimide-nucleophile modification of carboxyl groups in botulinum neurotoxin types A and E. manuscript submitted.

5. Sathyamoorthy, V., and DasGupta, B. R. Reductive methylation of lysine residues of botulinum neurotoxin type A and B. Mol. and Cell. Biochem. in press.

6. Woody, M. and DasGupta, B. R. Effect of tetranitromethane on the biological activities of botulinum neurotoxin types A, B and E. Mol. and Cell. Biochem. in press.

7. Datta, A., and DasGupta, B. R. Botulinum neurotoxin types A, B and E: pH induced difference spectra. Mol. and Cell. Biochem. 81, 187-194 (1988).

8. Datta, A., and DasGupta, B. R. Circular dichroic and fluorescence spectroscopic study of the conformation of botulinum neurotoxin types A and E. Mol. and Cell. Biochem., 79, 153 (1988).

9. Singh, B. R. and DasGupta, B. R. Structure of heavy and light chain subunits of type A botulinum neurotoxin analyzed by circular dichroism and fluorescence measurements. Mol. and Cell. Biochem. in press.

10. Montecucco, C., Schiavo, G., Gao, A., Bauerlein, E., Boquet, P., and DasGupta, B. R. Interaction of botulinum and tetanus toxins with lipid bilayer surface. Biochem. J. 251, 379 (1988).

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